

Appl. No. : **09/918,026**
Filed : **July 30, 2001**

REMARKS

Claims 1, 4-10, 12 and 13 are currently presented for examination. Claim 1 is currently amended. Support for the amendment can be found at page 12, lines 32-35, in Table 1 at page 87, in Table 2 at pages 88-89 and elsewhere throughout the specification. Accordingly, no new matter has been added to the instant application.

After careful consideration of the final Office Action mailed July 27, 2005, Applicants respectfully traverse the rejection of claims 1, 4-10, 12 and 13

Rejection of claims 1, 4-10, 12 and 13 under 35 U.S.C. § 112, first paragraph

The Examiner rejects claims 1, 4-10, 12 and 13 under 35 U.S.C. § 112, first paragraph as containing subject matter which was not described in the specification in such a way as to convey to a skilled artisan that the inventors possessed the claimed subject matter at the time of filing the instant application. In particular, the Examiner asserts that the amendment to claim 1 filed May 16, 2005 introduces new matter into the disclosure because there is allegedly no support in the originally-filed specification for the phrase "inhibits the expression of a nucleic acid molecule encoding acyl CoA cholesterol acyltransferase-2 by at least 60%."

Applicants maintain that claim 1 as amended May 16, 2005 is fully supported by the originally-filed specification; however, in order to expedite allowance of the instant application, Applicants currently amend claim 1 to recite the phrase "inhibits the expression of a nucleic acid molecule encoding acyl CoA cholesterol acyltransferase-2 by at least 55%." A review of Table 2 shows the antisense molecule of SEQ ID NO: 49 inhibits acyl CoA cholesterol acyltransferase-2 expression by 55%. Furthermore, within Tables 1 and 2 Applicants describe an additional eight antisense molecules that inhibit acyl CoA cholesterol acyltransferase-2 expression by greater than 55% up to 100%. As such, Applicants respectfully submit that the originally-filed specification fully demonstrates that the inventors possessed the subject matter recited in claim 1 at the time of filing the instant application.

In view of the foregoing remarks, Applicants respectfully request that the Examiner withdraw the rejection of claims 1, 4-10, 12 and 13 under 35 U.S.C. § 112, first paragraph.

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Rejection of claims 1, 4-10, 12 and 13 under 35 U.S.C. § 103(a)

The Examiner maintains the rejection of claims 1, 4-10, 12 and 13 under 35 U.S.C. § 103(a) as being obvious over Oelkers et al. in view of Chong et al. and Bennett et al. In particular, the Examiner asserts that Chong et al. motivates one of ordinary skill in the art to apply the general antisense teachings in Bennett et al. to prepare oligonucleotides complementary to the nucleic acid sequence encoding acyl CoA cholesterol acyltransferase-2, which is allegedly described in Oelkers et al.

Applicants respectfully submit that claims 1, 4-10, 12 and 13 are not obvious in view of the combination of Oelkers et al., Chong et al. and Bennett et al. In particular, Applicants would like to draw the Examiner's attention to the fact that under 35 U.S.C. § 103(c), it is not permissible to use Bennett et al. in a rejection of the instant claims under 35 U.S.C. § 103(a). Specifically, at the time of invention, the subject matter of the instant claims and the subject matter of Bennett et al. was owned by the same entity, and thus, Bennett et al. is not available for use as a 35 U.S.C. § 102(e)-type reference in the Examiner's obviousness rejection. As such, the subject matter of claims 1, 4-10, 12 and 13 is not obvious over Oelkers et al. in view of Chong et al. and Bennett et al.

However, even if Bennett were a proper prior art reference, Applicants respectfully submit that claims 1, 4-10, 12 and 13 would not be obvious over Oelkers et al. in view of Chong et al. and Bennett et al. because this combination does not teach or suggest specific inhibition of acyl CoA cholesterol acyltransferase-2. Applicants claim antisense oligonucleotides specific for acyl CoA cholesterol acyltransferase-2. In particular, claim 1 recites, in relevant part, "wherein said antisense oligonucleotide specifically hybridizes with said region and inhibits the expression of a nucleic acid molecule encoding acyl CoA cholesterol acyltransferase-2 by at least 55%." The meaning of the "specifically hybridizes" is set forth in the specification at pages 9-10. In particular, the specification states that "[a]n antisense compound is specifically hybridizable when . . . there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired." See page 10 at lines 7-10.

Prior to the filing of the instant application, it was not clear to those of ordinary skill in the art whether specifically inhibiting only one of the two known acyl CoA cholesterol

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acyltransferase isoforms would be more advantageous than inhibition of the other isoform. At that time, it was established that each of these isoforms mediated identical esterification reactions but the physiological role of these molecules was not clearly understood. The advantage of specifically inhibiting acyl CoA cholesterol acyltransferase-2 expression has been only recently elucidated. (See the enclosed copy of Rudel, et al. (2005) *Arterioscler Thromb Vasc Biol.* **25**:1-7, at abstract and at page 5). Current evidence now supports selective inhibition of the acyl CoA cholesterol acyltransferase-2 isoform because the production of cholesterol esters by this enzyme has been implicated in atherosclerosis. Additionally, the inhibition of the acyl CoA cholesterol acyltransferase-1 isoform has been recently shown to have toxic effects. (See Rudel, et al at abstract). Because no clear benefit to selectively inhibiting the activity of acyl CoA cholesterol acyltransferase-2 was known prior to the invention of the claimed subject matter, a skilled artisan would not have been motivated to produce the claimed antisense oligonucleotides which are specific for acyl CoA cholesterol acyltransferase-2.

In view of the foregoing remarks, Applicants respectfully submit that claims 1, 4-10, 12 and 13 are not obvious in view of the art of record. As such, Applicants respectfully request that the Examiner withdraw the rejection of claims 1, 4-10, 12 and 13 as obvious under 35 U.S.C. § 103(a).

CONCLUSION

Applicants believe that all outstanding issues in this case have been resolved and that the present claims are in condition for allowance. Nevertheless, if any undeveloped issues remain or if any issues require clarification, the Examiner is invited to contact the undersigned at the telephone number provided below in order to expedite the resolution of such issues.

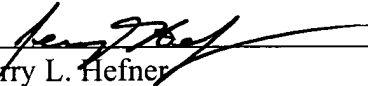
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Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: January 13, 2006

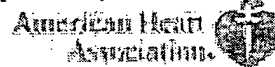
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ACAT2 Is a Target for Treatment of Coronary Heart Disease Associated With Hypercholesterolemia

Lawrence L. Rudel, Richard Lee, Paolo Parini

Abstract—The inhibition of intracellular cholesterol esterification as a means to prevent atherosclerosis has been considered to have potential for many years. Two different ACAT enzymes were discovered about 7 years ago, and it has become clear that the two enzymes provide separate physiologic functions. Much has been learned from mice with gene deletions for either ACAT1 or ACAT2. Deletion of ACAT2 has consistently been atheroprotective whereas deletion of ACAT1 has been varyingly problematic. ACAT1 functions in converting cellular cholesterol into cholesteryl ester in response to cholesterol abundance inside the cells. In atherosclerotic lesions, where macrophages ingest excess cholesterol, the ability to esterify the newly-acquired cholesterol seems important for cell survival. Inhibition of ACAT1 may bring undesired consequences with destabilization of cellular membrane function upon cholesterol accumulation leading to macrophage cell death. In contrast, ACAT2 is expressed only in hepatocytes and enterocytes, where ACAT1 is silent, and appears to provide cholesteryl esters for transport in lipoproteins. These two cell types have an abundance of additional mechanisms for disposing of cholesterol so that depletion of ACAT2 does not signal apoptosis. At the present time, the bulk of the available data suggest that the strategy seeming to bear the most potential for treatment of coronary heart disease associated with hypercholesterolemia would be to specifically inhibit ACAT2. (*Arterioscler Thromb Vasc Biol.* 2005;25:1-7.)



Key Words:

hypercholesterolemia, atherosclerosis, ACAT2

The inhibition of intracellular esterification of cholesterol as a means to prevent the arterial cholesteryl ester accumulation in atherosclerosis has been a strategy considered to have potential throughout the scientific community for many years. Approximately 7 years ago, 2 different ACAT enzymes were discovered, and in the intervening period of time, it has become clear that the 2 enzymes are localized in different cell types and provide separate physiological functions. Much key information has been learned from mice with gene deletions for either ACAT1 or ACAT2. Deletion of ACAT2 has consistently been atheroprotective,^{1,2} whereas deletion of ACAT1 has been varyingly problematic.³⁻⁵ ACAT1 functions in converting cellular cholesterol into cholesteryl ester in response to cholesterol abundance inside the cells. In atherosclerotic lesions, where macrophages ingest bound lipoproteins and even cellular fragments and lipid droplets, the ability to intracellularly esterify much of the newly acquired cholesterol seems important for cell survival.⁶ Inhibition of ACAT1 may bring undesired consequences with destabilization of cellular membrane function on cholesterol accumulation, and cell death as has been identified in macrophages from ACAT1 knockout mice.⁷ Although smooth muscle cells may not undergo apoptosis on

inhibition of ACAT1⁸ and thus could serve to rescue some aspects of lesion remodeling on inhibition of ACAT1 and loss of macrophage function,⁹ this eventuality is purely hypothetical at this time and does not explain the atherosclerosis of ACAT1 knockout mice. In contrast, ACAT2 is expressed only in hepatocytes and enterocytes,¹⁰ cells where ACAT1 is silent, and appears to function, at least in part, by providing cholesteryl esters for transport in lipoproteins. These cell types have an abundance of additional mechanisms for disposing of cholesterol so that depletion of ACAT2 does not signal apoptosis. At the present time, the bulk of the available data suggest that the strategy seeming to bear the most potential for treatment of coronary heart disease associated with hypercholesterolemia would be to specifically inhibit ACAT2.

Factors Distinguishing ACAT1 From ACAT2

Physical Orientation

Whereas ACAT1 and ACAT2 catalyze the same chemical reactions, the apparent physical structure of the enzyme protein and the physiological roles of these 2 enzymes in cholesterol metabolism are distinct from one another. Our understanding of these roles has progressed considerably over

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the past 7 years, although the enzymes are ER proteins that continue to resist purification, leaving many questions remaining about function. Studies from 2 laboratories have been completed that indicate that of the computer-predicted 7 or 8 transmembrane domains for ACAT1 and ACAT2, only some of them are used and extend across the ER membrane. In the first published study,¹¹ 5 transmembrane domains for ACAT1 were used and 3 of the same 5 transmembrane domains for ACAT2 were used, as were 2 others, for a total of 5 for ACAT2 as well. In studies from the Chang laboratory, 7 transmembrane domains were predicted to be used for ACAT1, whereas only 2 were predicted to be used in ACAT2.^{12,13} Different methodology was used by the 2 groups, and it is not yet clear which model for the insertion of these enzyme proteins into and across the membrane might be correct. In any case, data from both laboratories supporting fundamental structural differences gave hope that it would be possible to find compounds that are fully specific for inhibition of ACAT1 versus ACAT2. In fact, in 2004, we showed that the compound pyripyropene A had >2000-fold higher specificity for ACAT2 compared with ACAT1 in a cell-based assay.¹⁴ Although pyripyropene A is not a drug, this observation establishes proof of concept for selective inhibition.

Tissue Distribution

A difference that provides insight into defining physiological functions is the unique cellular distribution within and among the tissues of the body. Whereas ACAT1 is located in at least 1 cell type in most tissues of the body,¹⁵⁻¹⁷ the expression of the ACAT2 protein is limited to only 2 cell types, the small intestinal enterocytes and hepatocytes.¹⁸ Studies on the 5'-untranslated region of the human ACAT2 have identified HNF1 as one of the main transcription factors responsible for the expression of this enzyme in hepatocytes (Pranfolk and Parini, submitted manuscript). Interestingly, our data suggest that both ACAT1 and ACAT2 are not expressed in a physiological setting in the same cell type in any tissue of the body. It is possible to find both ACAT1 and ACAT2 in HepG2 cells and in CaCo₂ cells, and some have claimed that isolated hepatocytes may contain both ACAT enzymes,¹⁵ and macrophages may express both,¹⁹ but the best evidence supports the fact that in mammals, enterocytes and hepatocytes are the only 2 cell types to abundantly express ACAT2, and these are cells that do not express detectable amounts of ACAT1. This finding suggests that mechanisms exist to signal the expression of ACAT2 and simultaneously silence ACAT1 and vice versa.

For several years, the only available ACAT1 and ACAT2 localization data in humans claimed that ACAT2 was not located in the hepatocytes of the adult liver.^{18,20} However, analyses had been performed on postmortem tissue that may have experienced from degradation during the postmortem interval. Data were shown in isolated hepatocytes, but without documentation that cell preparations were purely hepatocytes, and the data on immunostaining did not clearly delineate which of the ACAT isoforms resided within hepatocytes versus other cell types. In a recent collaboration with the Bo Angelin laboratory at the Karolinska Institute in Stockholm, Sweden, we have been able to obtain snap-frozen

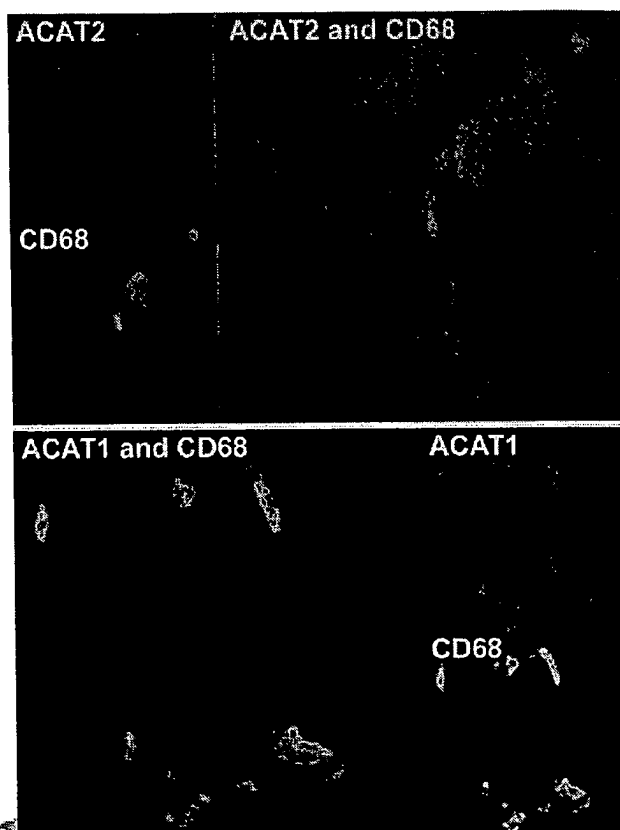


Figure 1. Immunolocalization of ACAT2, CD68, and ACAT1. A liver biopsy sample was collected from a female gallstone patient and evaluated as described previously.²¹ Sections from frozen tissue were fixed in acetone. Upper panels, Rhodamine channel (red) image of the section stained with anti-ACAT2 antibody, fluorescein isothiocyanate (FITC) channel (green) image of the section stained with anti-CD68 (marker for Kupffer cells), and overlay of rhodamine channel and FITC channel showing localization of ACAT2 and CD68 in different cell types. Lower panels, Overlay of rhodamine channel and FITC channel showing colocalization of ACAT1 and CD68 in the Kupffer cells, rhodamine channel image of the section stained with anti-ACAT1 antibody, and FITC channel image of the section stained with anti-CD68.

human liver biopsy specimens taken at the time of surgery for gallstone removal. Immunofluorescence analysis of these samples clearly showed that ACAT2 was expressed in the ER of all hepatocytes, whereas ACAT1 was only detected within Kupffer cells²¹ (Figure 1). Curiously, the relative abundance of mRNA for ACAT1 and ACAT2 from the human liver samples was similar, although ACAT2 was found to provide the majority of the immunostaining and cholesterol esterification activity. The latter outcome suggests that the mRNA production or stability for the 2 enzymes may not be the same or that transcriptional efficiency and/or protein stability of the 2 enzymes is different. More work will have to be performed to find the correct explanation, but at the present time, estimates of the relative functional amounts of the 2 enzymes are better made based on activity.

Cellular Function

The function of ACAT2 in the hepatocyte is to provide esterified cholesterol for incorporation into very-low-density

lipoprotein (VLDL), as well as to provide cholesteryl ester for cytoplasmic lipid droplets, a means for storage when liver cholesterol is abundant. Our data have demonstrated that increased VLDL cholesteryl ester secretion occurs in livers of monkeys fed dietary cholesterol.²² In these animals, liver perfusion studies showed that the rate of cholesteryl ester accumulation during recirculating perfusion was highly correlated to the extent of coronary artery atherosclerosis. This finding suggests that the ACAT2-derived cholesteryl esters that accumulate in the apolipoprotein B-containing lipoproteins during atherogenic diet feeding, primarily cholesteryl oleate, promote arterial cholesterol accumulation during atherogenesis. A correlation has been consistently noted between increased low-density lipoprotein (LDL) particle size and enhanced atherosclerosis²³ that may be the result of ACAT2-mediated cholesteryl oleate accumulation in plasma LDL.²⁴ Because the only form of ACAT seen in monkey hepatocytes is ACAT2, the secreted lipoprotein cholesteryl ester is likely derived from ACAT2. We tested this hypothesis in ACAT2^{-/-} mice in which the perfused liver cholesteryl ester accumulation rate was <5% of that seen in wild-type mice demonstrating that hepatic lipoprotein cholesteryl ester originates from ACAT2 (Lee and Rudel, manuscript submitted).

That these findings are highly relevant to humans is indicated by situations in which dietary cholesterol and saturated fat feeding result in larger LDL particles.^{25,26} The authors of these manuscripts focused on the potential increase in atherogenicity of small dense LDL. However, the likelihood that saturated fat-rich diet induces accumulation of larger, more cholesteryl ester-rich LDL probably as a result of increased hepatic secretion of ACAT2-derived cholesteryl esters, seems more likely to indicate a state of increased, not decreased, atherogenicity analogous to that repeatedly documented in studies in nonhuman primates.²⁷ In addition, several epidemiologic studies have shown that larger LDLs are risk factors for coronary heart disease in humans,²⁷⁻³⁰ and several publications have shown that a higher proportion of cholesteryl linoleate among plasma cholesteryl esters is associated with a reduced incidence of complications from coronary heart disease.³¹⁻³⁴ Cholesteryl linoleate is typically derived from esterification of cholesterol by the plasma enzyme lecithin:cholesterol acyltransferase (LCAT), whereas the primary cholesteryl ester products of ACAT2 are cholesteryl oleate and cholesteryl palmitate. These observations suggest that coronary heart disease patients compared with healthy controls had lower proportions of LCAT-derived cholesteryl linoleate and, by difference, higher proportions of ACAT2-derived cholesteryl ester. Although the evidence in humans is necessarily circumstantial, there are numerous indications that ACAT2-derived cholesteryl esters accumulate in plasma in association with increased coronary heart disease. In monkeys and in mice, this accumulation has been highly correlated with increased atherosclerosis.

ACAT2 in the enterocyte has been hypothesized to play an important role in the absorption of cholesterol into the body as it esterifies newly absorbed dietary cholesterol for incorporation of the cholesteryl ester product into the core of chylomicron particles subsequently secreted into the circula-

tion.^{35,36} Analysis of chylomicrons secreted into the lymph of rabbits and monkeys showed that $\approx 75\%$ of the cholesterol was esterified.^{35,37} If it were assumed that all the esterified cholesterol is ACAT2-derived, the absence of the enzyme should lead to a 75% decrease in the absorption of cholesterol. However, cholesterol absorption in ACAT2^{-/-} mice only decreased by 30% to 40%, although the amount of cholesterol absorbed was a lesser proportion as the amount of dietary cholesterol was increased.³⁸ Whereas ACAT2-dependent absorption of cholesterol certainly occurs in a normal enterocyte, the data suggest that compensatory mechanisms exist in the enterocyte that maintain cholesterol absorption in the absence of ACAT2. It is possible that the chylomicron surface is enriched in free cholesterol that does not get incorporated as cholesteryl ester into the core of the particle.³⁹ However, the degree to which such a process can compensate for the loss of ACAT2 is uncertain. Another intriguing compensatory mechanism was suggested when the ABCA1 mRNA levels in the enterocytes of the ACAT2^{-/-} mice were quantified, and it was found that their levels were 3-fold greater than the levels found in wild-type mice.^{38,40} In CaCO₂ cells, a role for ABCA1 in compensating for cholesterol transport in chylomicrons has been suggested.⁴¹ This led us to hypothesize that in the absence of ACAT2, cholesterol is transported to the basolateral side of the enterocyte, where it undergoes ABCA1 mediated efflux out of the cell onto high-density lipoprotein (HDL) particles. Despite the fact that this effect would increase the amount of dietary cholesterol absorbed, it could be an anti-atherogenic process because the cholesterol would be immediately incorporated onto the HDL particles, and these lipoproteins could serve to increase reverse cholesterol transport. Stimulation of intestinal ABCA1 would provide a second mechanism by which ACAT2 inhibition can elevate HDL cholesterol levels, with the other being the increase in LCAT activity demonstrated in the ACAT2^{-/-} apolipoprotein E^{-/-} mouse study.¹

Although differences in the regulation of expression of the 2 ACAT enzymes have been demonstrated, some of the functions must be similar. For example, when cholesterol is available in excess, hepatocytes are known to accumulate cholesteryl esters in lipid droplets, a process appearing analogous to that in macrophages during foam cell formation. Studies also suggest that either isoform can be artificially expressed in cells and still performed the ACAT-specific functions necessary for that cell type. For instance, transient expression of either ACAT2 or ACAT1 in AC-29 cells (SCAP-mutant Chinese hamster ovary cells without ACAT activity⁴²) leads to the development of cytoplasmic cholesteryl ester droplets,¹⁴ demonstrating that both ACATs function in intracellular cholesterol homeostasis. Conversely, adenoviral expression of ACAT1 in the liver of hamsters led to the elevation of total plasma cholesterol concentration,⁴³ suggesting that with overexpression, ACAT1 was capable of incorporating cholesteryl esters into apolipoprotein B-containing lipoproteins. Taken together, the data suggest that the unique cellular expression patterns in tissues for ACAT1 and ACAT2 are characteristics that provide unknown uniqueness in the functions of these enzymes.

Sterol Specificity

It has been demonstrated that cholesterol and plant sterols are transported into the enterocyte, presumably through NPC1L1.⁴⁴ However, once in the enterocyte, ~50% of dietary cholesterol is absorbed into the circulation, whereas <5% of the dietary plant sterols are absorbed. The recent discovery that ACAT2, but not ACAT1, preferentially esterifies cholesterol and not plant sterols suggests another possible role for ACAT2 in the enterocyte, ie, that of "gatekeeper," limiting the absorption of sitosterol.⁴⁵ It is possible that once in the intestine, sterols are transported to the ER, where ACAT2 preferentially esterifies cholesterol. The remaining unesterified plant and animal sterols would then be targeted for efflux back into the intestinal lumen by ABCG5/G8. More studies are needed to develop a better understanding of the role of ACAT2 in the absorption of both cholesterol and plant sterols in the small intestine.

Associations of ACAT1 and ACAT2 With Atherosclerosis

Studies in ACAT1 Knockout Mice

In 1996, the production of genetically engineered mice lacking functional ACAT1 was first reported by Farese et al.³ ACAT1 gene disruption resulted in decreased cholesterol esterification in fibroblasts and adrenals. In contrast, the livers of ACAT-deficient mice contained substantial amounts of cholesteryl esters and exhibited no reduction in cholesterol esterification activity, providing some of the first evidence of the presence of a second cholesterol esterifying enzyme. Intestinal cholesterol absorption was normal in ACAT1^{-/-} mice. The decreased content of cholesteryl esters in macrophages from ACAT1^{-/-} mice and their reduced ability to accumulate cholesteryl esters on incubation with acetylated LDL suggested the hypothesis that protection against development of atherosclerosis might result from the absence of ACAT1 activity.

Subsequently, ACAT1^{-/-} mice were crossed with 2 genetically engineered mouse strains susceptible to atherosclerosis, apolipoprotein E-deficient (apoE^{-/-}) and LDL receptor-deficient (LDLR^{-/-}) mice.⁴⁶ The marked alteration in cholesterol homeostasis caused by ACAT1 deficiency led to massive deposition of unesterified cholesterol in the skin and in the brain. The atherosclerotic lesions in apoE^{-/-} and in LDLR^{-/-} mice lacking ACAT1 had reduced levels of neutral lipids and a paucity of macrophages in the more advanced lesion, apparently reflecting progressive cell death possibly from free cholesterol toxicity analogous to that previously observed in vitro.^{6,46,47} Subsequently, bone marrow transplantation was adopted to introduce ACAT1^{-/-} macrophages into LDLR^{-/-} mice.⁷ Contrary to previous hypotheses, LDLR^{-/-} mice receiving ACAT1^{-/-} macrophages developed larger atherosclerotic lesions despite unchanged plasma lipid levels. Thus, ACAT1 deficiency in macrophages appeared to promote rather than prevent atherosclerotic lesion development in mice with congenital hyperlipidemia. A detailed in vitro study on macrophages has recently shown that ACAT1 deficiency induces free cholesterol accumulation and disrupts the efflux of cellular cholesterol, despite increased expression

of ABCA1 and increased efflux of lipoprotein-derived cholesterol.⁴⁸ Intracellular vesicle accumulation was seen that could be the origin of increased extracellular cholesterol in atherosclerotic lesions of ACAT1^{-/-} mice, suggesting additional mechanisms for increased atherosclerosis that may occur in ACAT1^{-/-} mice.

Studies in ACAT2 Knockout Mice

The discovery of ACAT2 was stimulated by the studies in ACAT1^{-/-} mice showing that plasma and liver cholesteryl ester concentrations remained relatively unchanged in the absence of ACAT1, suggesting an additional enzyme. The identification of an ACAT-related gene family in yeast occurred at approximately the same time,⁴⁹ establishing a precedent for >1 ACAT enzyme. Cloning of a second ACAT, termed ACAT2, soon followed in mice, monkeys, and humans.¹⁵⁻¹⁷ Generation of mice with an ACAT2 gene deletion provided the opportunity for the role of this enzyme to be better-defined.³⁹ Using this model, the role of ACAT2 as the principal cholesterol esterifying enzyme in small intestine and in the liver was demonstrated. Mice deficient for ACAT2 had almost a complete absence of cholesterol esterifying activity in these 2 organs, and although the phenotype was unremarkable when the mice were fed chow diets, major differences were apparent when the Paigen diet containing cholesterol, saturated fat, and cholic acid was fed. A clear resistance to hypercholesterolemia, a reduction in cholesterol gallstones, and a decrease in intestinal cholesterol absorption were evident in the mice lacking ACAT2.

The development of atherosclerosis in ACAT2 deficiency was first studied in apolipoprotein E/ACAT2 double-knockout mice.¹⁴ Despite similar concentrations of plasma apolipoprotein B, total plasma cholesterol was decreased in apoE^{-/-} ACAT2^{-/-} mice compared with the apoE^{-/-} control mice, caused principally by the >70% decrease in plasma cholesteryl esters. After 27 weeks on chow diets, female apoE^{-/-} ACAT2^{-/-} mice had greatly reduced levels of aortic atherosclerosis compared with the apoE^{-/-} ACAT2^{+/+} controls. The lipid core of plasma apolipoprotein B-containing lipoproteins in apoE^{-/-} ACAT2^{-/-} mice consisted primarily of triglycerides rather than cholesteryl esters, whereas the plasma levels of apolipoprotein B were not lowered as much. These data suggest that the number of apolipoprotein B-containing lipoproteins in plasma, per se, is not the critical factor in the development of atherosclerosis. Rather, the amount of ACAT2-derived cholesteryl ester present in the core of these lipoproteins is apparently more important. Although these apoE^{-/-} ACAT2^{-/-} mice did not show hypertriglyceridemia, ACAT2-derived cholesteryl esters were, for the most part, replaced by triglycerides in the ACAT2^{-/-} mice and, with reductions in the extent of atherosclerosis, the data demonstrate that triglycerides apparently have a lower atherogenic potential than the replaced cholesteryl esters.

Further evidence for the atherosclerosis promoting activity of ACAT2 comes from studies in LDLR^{-/-} mice in which ACAT2 and LCAT were genetically disrupted.² These groups of mice were fed a diet with 10% of energy as transmonounsaturated-enriched fat and 0.18% cholesterol for 20 weeks. As was found in the apoE^{-/-} ACAT2^{-/-} mice, LDLR^{-/-}

ACAT2^{-/-} mice had greatly reduced aortic atherosclerosis (>80% reductions in aortic lesion surface area and cholesteryl ester concentration) compared with the LDLR^{-/-} ACAT2^{+/+} controls. Deletion of both ACAT2 and LCAT in LDLR^{-/-} mice resulted in a dramatic reduction in atherosclerosis and a complete absence of plasma cholesteryl esters, firmly establishing that ACAT1 does not contribute to the synthesis of plasma cholesteryl esters in mice. LCAT deficiency led to increased atherosclerosis in this experiment because the LCAT^{-/-}LDLR^{-/-} mice had higher total plasma and VLDL cholesterol attributable to an increase in ACAT2-derived cholesteryl esters (cholesteryl oleate and cholesteryl palmitate) together with lower LDL and HDL cholesterol concentrations. Hence, beyond HDL deficiency and reduced reverse cholesterol transport in LCAT deficiency, the modification of cholesteryl ester concentration and composition in apolipoprotein B-containing VLDL and LDL of LCAT^{-/-}LDLR^{-/-} mice likely contributed to the higher extent of atherosclerosis observed in these animals. Likewise, the decrease in atherosclerosis seen in LDLR^{-/-} mice deficient in ACAT2 corroborates the hypothesis that the plasma cholesteryl esters synthesized by ACAT2 are atherogenic. Even though LDL cholesterol concentrations were not decreased, the plasma apolipoprotein B-containing lipoprotein cholesteryl esters of ACAT2^{-/-}LDLR^{-/-} mice were all derived from the activity of LCAT and mainly consisted of polyunsaturated fatty acid-containing cholesteryl esters, i.e., not the cholesteryl oleate and cholesteryl palmitate synthesized by ACAT2.

Studies in Monkeys

A role for ACAT2 in diet-induced atherosclerosis in monkeys has recently become clear. For many years, it was recognized that plasma LDL that are enriched in cholesteryl esters accumulate in the plasma of monkeys that are more susceptible to coronary artery atherosclerosis.²⁴ The enrichment of LDL with cholesteryl oleate and cholesteryl palmitate, specifically, was noted. Subsequently, observations were made that during liver perfusion, the secretion rate of cholesteryl esters was highly correlated to the extent of coronary artery atherosclerosis and to the cholesteryl ester enrichment of plasma LDL.²² Dietary fats that promoted the accumulation of cholesteryl oleate in plasma LDL particles, such as saturated and monounsaturated fatty acid-rich oils, were shown to promote atherosclerosis to a greater extent than polyunsaturated fats.²³ Although both ACAT1 and ACAT2 were identified in nonhuman primate liver,¹⁵ immunolocalization showed that the only ACAT enzyme in hepatocytes within primate liver was ACAT2; ACAT1 was present in Kupffer cells.¹⁰ Primate species comparisons showed that cynomolgus monkeys hyper-responsive to atherogenic diets had more ACAT2 protein and activity than hypo-responsive African green monkeys.⁵⁰ Therefore, the data are consistent in supporting the likelihood that increased ACAT2-mediated synthesis and secretion of cholesteryl oleate in nonhuman primates leads to increased atherogenicity. At this point, the critical experiment remaining that would close the loop even more tightly around ACAT2 as the source of atherogenic plasma cholesteryl esters in nonhuman primates would be an

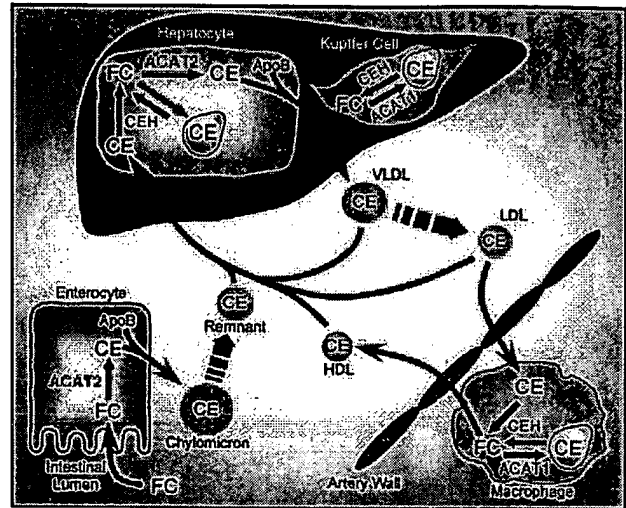


Figure 2. Diagram of the separate roles of ACAT1 and ACAT2 in cholesterol metabolism in the liver, enterocyte, and macrophages within the artery wall. Cell types with ACAT2 are the enterocyte and the hepatocyte, which are the lipoprotein secreting cells of the intestine and liver, respectively. The Kupffer cells of the liver and macrophages of the artery wall are scavenger cells that actively engulf many forms of cholesterol. These cells express ACAT1, which appears to function in maintenance of free cholesterol levels appropriate for cell membrane function.

experiment in which direct inhibition of ACAT2 was linked to atherosclerosis prevention. This experiment will have to await the development of ACAT2-specific inhibitors. Nevertheless, currently available data strongly point to ACAT2 as a major contributor to the diet-induced coronary artery atherosclerosis in nonhuman primates.

Conclusions

The current state of our understanding about the contrasting roles of ACAT1 and ACAT2 in cholesterol metabolism in the body is illustrated in Figure 2. ACAT2 functions in cholesterol esterification (FC) during cholesterol absorption in the enterocyte. Cholesterol is transported into the cell, where some of it is esterified by ACAT2, incorporated into chylomicrons, and transported primarily as CE into the body. The metabolism of chylomicrons in the circulation leads to the formation of remnant lipoproteins that retain the CE, which is ultimately delivered to the hepatocytes in the liver. Hydrolysis of the cholesteryl ester by a cholesteryl ester hydrolase follows uptake and some of the resulting free cholesterol becomes available for re-esterification by ACAT2. The cholesteryl ester so derived may be incorporated into lipid storage droplets in the cytoplasm (from which FC can be regenerated) or incorporated into lipoproteins together with apolipoprotein B during VLDL particle assembly and secretion. Cholesteryl ester secreted in VLDL becomes part of the cholesteryl ester in LDL (LCAT also provides some of the cholesteryl ester in LDL) or can potentially be cleared from plasma by return to the hepatocyte. The cholesteryl ester in LDL can also be transferred among lipoproteins by CETP or can be filtered into tissues such as the arterial intima, where it is eventually taken up into macrophages and hydrolyzed to FC. The FC is either effluxed out of the cell or, in situations

in which cholesterol is present in excess, such as during developing atherosclerosis, it may be resynthesized by ACAT1 and stored in lipid droplets. The process of synthesis and hydrolysis of cholesteryl ester is dynamic and when many of these droplets are formed, the macrophages become known as foam cells. Thus, ACAT1 has a key role in the development of early atherosclerotic lesions in that it generates the cholesteryl ester of the foam cell, albeit in response to excessive cholesterol availability within the cell. Cholesterol that is effluxed from cells such as the macrophage appears in the circulating HDL, where it can be esterified by the plasma enzyme LCAT for the eventual return of the cholesteryl ester to the hepatocytes in the liver.

Therefore, the role of ACAT1 is to maintain the appropriate FC levels inside cells, presumably in response to the needs of membrane function. The presence of this enzyme in scavenger cell populations appears particularly critical in that through their normal function, these cells can become loaded with excessive amounts of cholesterol. Limiting the role of ACAT1 in these cells may contribute to a decline of cell function resulting in many consequences, not all of them beneficial. Interestingly, the 2 cell types that normally respond to even greater demands for cholesterol processing, the enterocyte and the hepatocyte, express a different form of the cholesterol esterifying enzyme, ACAT2. This enzyme functions to provide cholesteryl ester for secretion in lipoproteins as well as for storage within cytoplasmic lipid droplets. We assume that nature's choice to use a different enzyme protein for cholesterol esterification in these cells is related to the additional function of providing cholesteryl ester for lipoprotein secretion; however, at this time, this concept is only hypothetical. Nevertheless, given the uniqueness in cellular distribution, selective inhibition of ACAT2 could provide benefits in decreasing plasma cholesteryl ester availability. The general good health of the ACAT2 knockout mouse suggests that the enterocyte and hepatocyte have sufficient backup pathways for cholesterol metabolism that adequately compensate for the absence of this enzyme, a situation not shared by the ACAT1 knockout mouse. When all of the information available at this time is combined, it appears that it would be beneficial to identify selective inhibitors of ACAT2 for treatment of premature coronary artery atherosclerosis. Without ACAT2, we can expect a decreased atherogenicity of plasma lipoproteins while most of the cell types throughout the body retain normal membrane function because of continued availability of adequate ACAT1 function.

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